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Original article

Role of cyclins A and E in endometrial carcinogenesis in breast cancer patients under tamoxifen treatment

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KEYWORDS

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Abstract *Purpose:* The objective of our study was to determine the relevance of cyclins A and E overexpression in endometrial carcinogenesis in hormone receptor-positive breast cancer patients under tamoxifen therapy.
Experimental design: We assessed expression of cyclins A and E in Endometrial cytology samples collected from 36 ER and PR positive breast cancer patients; under tamoxifen treatment by using the Tao-brush non-invasive brushing cytology technique. Cyclins were detected in the collected samples by means of immuno-cytochemistry.
The patients included in this study are a cohort of 36 breast cancer patients who were operated upon at the National Cancer Institute – Cairo University in the period from February 2006 to

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May 2008 and received tamoxifen (TAM) as part of their adjuvant treatment.

Results: Cyclins A and E were expressed in 17 and 15 of the 36 collected endometrial cytology samples (47.2% and 41.6% respectively).

Expression of cyclins A and E was highly correlated to Tamoxifen exposure duration (32 and 43 months respectively) $p < 0.001$. Tamoxifen median exposure duration was shortened to 21 months in cases showing positivity for either markers, while in cases showing positivity for both cyclins, the median exposure duration was longer (44.5 months) ($p < 0.001$). Neither cyclin A nor E was detected before median tamoxifen exposure duration of 11 months. Endometrial carcinoma cases had the longest Tamoxifen exposure duration (60 months).

Conclusion: Cyclins A and E expression is involved in the carcinogenesis of endometrium in women with breast cancer and under tamoxifen-treatment. Follow up of the patients using these 2 markers is highly recommended starting from the 12th month.

Introduction

Tamoxifen is the first-choice adjuvant treatment for primary estrogen receptor-positive (ER+) breast cancer in postmenopausal women. It has been shown that survival rates in tamoxifen-treated women are improved as much as 50% [1]. Furthermore, tamoxifen use has also been shown to reduce the incidence of breast cancer in healthy women at a high risk for this disease [2–4]. The mechanism of action of tamoxifen in breast cancer patients is that it inhibits cancer cell growth by competitive antagonism with estrogen for its receptor [5].

One of the most significant side effects of treatment with tamoxifen appears to be its proliferative effect on the endometrium (estrogen-agonistic effect) [6]. Several studies have evaluated the incidence of endometrial pathologies in tamoxifen users, and although occurrence rates differ per study, a higher incidence in the tamoxifen group is generally agreed on. Endometrial pathologies associated with tamoxifen use include hyperplasia, polyps, carcinomas and sarcomas [7].

Current tools for endometrial evaluation are limited. Endometrial assessments are generally based exclusively on the histological appearance using eight morphologic markers originally proposed by Noyes, Rock and Hertig [8]. However, this classification has been shown to have limitations, with high inter- and intra-observer variation. Over time, incremental improvements and modifications have been performed on this classification on endometrial assessment. In addition to these modifications, the use of markers has been investigated as a way to better assess development and receptivity.

Tamoxifen induces a wave of DNA synthesis in the endometrial epithelial cells. The uterine epithelial cell proliferation is preceded by the mobilization of cyclin D1 from the cytoplasm to the nucleus which, together with CDK4, phosphorylates members of the Rb-retinoblastoma family of proteins, pRb and p107. Subsequent to this initial nuclear accumulation of cyclin D1, cyclin E and then cyclin A are induced that, together with the activation of CDK2, result in enhanced cyclin E- and cyclin A-dependent CDK2 kinase activity and further phosphorylation of pRb and p107.

Biomarkers have become of interest as potential predictors for outcome of adjuvant tamoxifen therapy [9]. Cyclins, their associated cyclin-dependent kinases, and cyclin-dependent kinase inhibitory proteins play a central role in the cell cycle progression and may also affect response to tamoxifen [9].

Cyclins A and E are considered potential candidate biomarkers in cancer [9]. Cyclin E has been shown to be the rate-limiting activator of the mitotic G1 to S phase transition.

While over expression of cyclin-A which regulates the S-G2-M phase transition of the cell cycle correlates closely with clinico-pathologic parameters and prognosis in patients with endometrial carcinoma.

The aim of the present study was to determine the role of the cell cycle regulators cyclins A and E overexpression in the carcinogenesis process of the endometrium in hormone receptor-positive breast cancer patients under tamoxifen therapy Figure 1–5.

Patients

The patients included in this study are a cohort of 36 breast cancer patients who were operated at the national cancer institute – Cairo University in the period from February 2006 to May 2008. Out of the 36 patients, 12 refused to participate in the study and 4 cases were excluded as their samples were inadequate.

Inclusion criteria:.

1- Histologically diagnosed breast cancer cases had an estrogen and progesterone hormonal receptor positive profile as a must positive inclusion criterion.

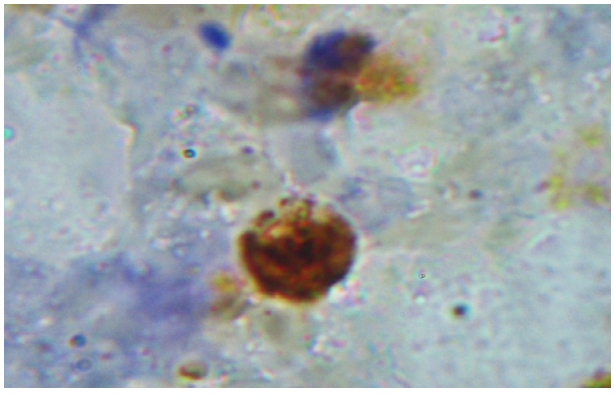

Figure 1 Immunostaining to cyclin E (×1000).

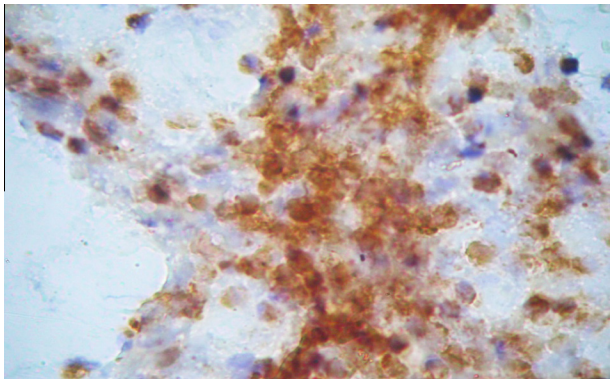

Figure 2 Immunostaining to cyclin A (×400).

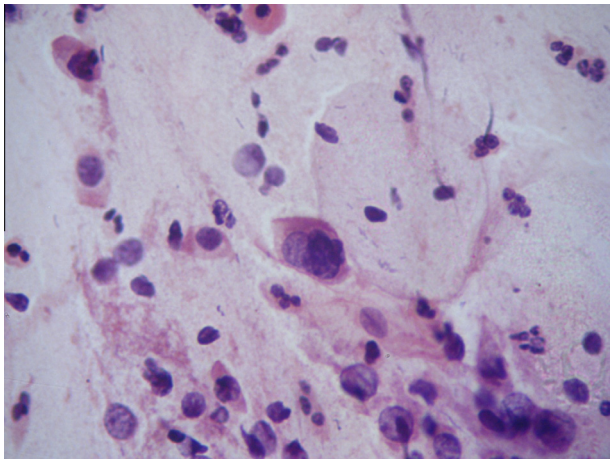

Figure 3 Atypical glandular cells of undetermined significance (AGUS).

2- All selected patients had post mastectomy radiotherapy and/or chemotherapy and were kept under Tamoxifen treatment at a dose of 20 mg/day up to 5 years according to their treatment protocol.

Our selected group of patients were exposed to Tamoxifen at different periods ranging from 5 to 60 months at the time of sampling retrieval.

Complete medical history and physical examination work-up were done before enrolling patients in this study.

1- Complete blood picture.

2- Blood chemistry, urea, creatinine, uric acid, bilirubin, SGOT, SGPT, Alkaline phosphatase, total protein, albumin, PT, PTT, Na, K

3- Chest X-ray, bone scan, abdominal sonar, CT scan and echocardiography when indicated.

4- Confirming the ER and PR by immunohistochemical staining (only positive estrogen and progesterone receptor patients were included).

History included also, revision of all prognostic factors at the time of diagnosis including: tumor size, grade, histological type, and lymph node involvement, type of treatment they received (surgery, post-operative radiotherapy or adjuvant chemotherapy).

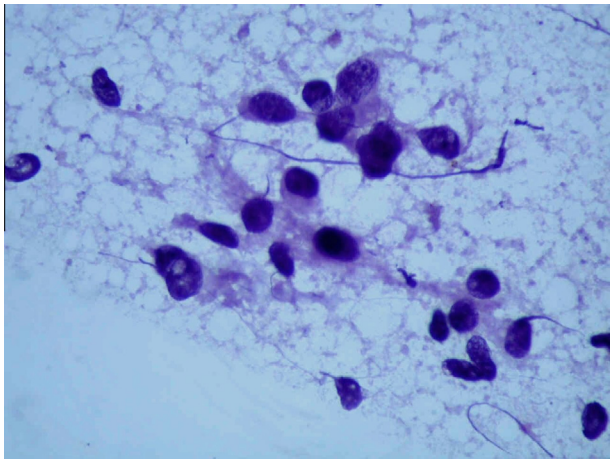

Figure 4 Atypical endometrial cells suggestive of adenocarcinoma.

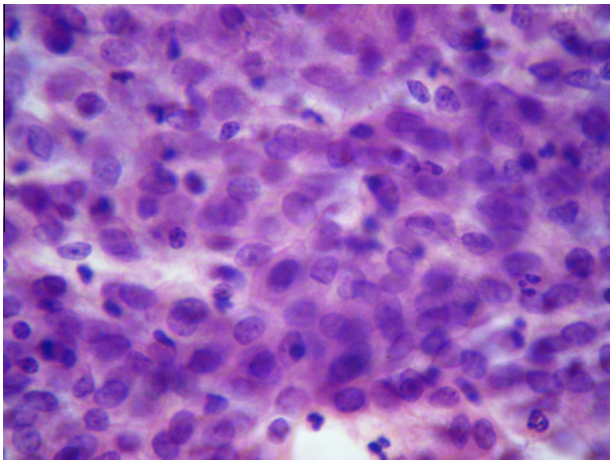

Figure 5 High power view of cells suggestive of adenocarcinoma.

Methods

Endometrial cytology using the endometrial cytology Tao- brush non-invasive technique was used for discovering and diagnosing malignant and premalignant states of the endometrium according to the criteria described by DeMay [10].

Endometrial cytology samples were collected from the selected 36 breast cancer patients; by using the endometrial cytology Tao- brush non-invasive technique. The recovered cytology samples were subjected to immunocytochemistry study using antibodies against cyclin A and cyclin E. All immunocytochemistry results were correlated with the routine cytology using the PAP stain.

The immunochemistry method for staining the recovered cytology sample with the biomarker cyclins A and E: The ethanol–fixed cytology slides were stained using the Imuno-cytochemical staining; performed using the DAKO LSAB kit (Labeled streptavidin Biotin) with mouse anti human cyclin A and mouse anti human cyclin E.

Immunocytochemistry was done and evaluated at the National cancer Institute –Cairo University by means of a standard protocol.

All immunochemical staining included positive and negative control.

Statistical methods

SPSS version 12.0 (Chi IL, USA) was used for data management and analysis. Median and range described quantitative data. Mann–Whitney *U* test compared means of 2 independent groups and Kruskal Wallis test compared more than 2 independent groups. All tests were two-sided. P-values $\leqslant 0.05$ were considered significant.

Results

The mean age at diagnosis was 45.4 (±7.73) mostly post-menopause (ranging between 43 and 65 years).

Breast cancer patients subjected to surgery were mostly of the aggressive and invasive form.

The mean tumor size- diameter was approximately 4.5 cm (±2.3). The relative frequency of their histological types showed invasive duct carcinoma to be 80.6% (29/36) of all studied patients, (11.1%) 4/36 were lobular + duct carcinoma while mucinous carcinoma were 3/36 (8.4%).

Axillary lymph node dissection showed metastasis in 21/36 (58.3%). The TNM staging showed predominance of stage II 58.3% 21/36 cases, followed by the GIII 25%, 9/36 and 6/36 samples (16.7%) were GI.

Smears showed cytological criteria as follows: Atypical Glandular cells of undetermined significance (AGUS) 11/36 (30.6%), Atrophy 10/36 (27.7%.).Reactive inflammatory changes 12/36 (33.3%). One case had atypical precancerous changes (2.8%) and 2 cases had endometrial carcinoma (5.6%). (Table 1).

Immuno-reactivity of the collected endometrial samples to Cyclin-A: showed 47.2% (17/36) positivity for cyclin A. However, 15 out of 36 cases were immune reactive for cyclin E (Table 1).

Table 1 Cytological results and cyclin immune-reactivity of the collected endometrial samples of the 36 breast cancer patients.

Results	Number	Percent
Cytological type		
AGUS	11	30.6
Atrophy	10	27.7
Reactive inflammatory changes	12	33.3
Atypical precancerous changes	1	2.8
Endometrial carcinoma	2	5.6
Cyclin A		
+ve	17	47.2
−ve	19	52.8
Cyclin E		
+ve	15	41.7
−ve	21	58.3
Total	36	100%

Table 2 Relation of the cytological type and cyclin immunoreactivity of the endometrial samples to tamoxifen exposure duration in the 36 breast cancer patients.

Results	Number	Median TAM. Duration/month	Range	*p* Value
Cytological Type				
AGUS	11	18(5–51)	46	
Reactive Inflammatory	12	17(5–52)	47	0.09
changes Atrophy	10	15.5(7–45)	38	
Cyclin A				
+ve	17	32(11–60)	49	
−ve	19	12(15–41)	36	<0.001
Cyclin E				
+ve	15	43(18–60)	42	
−ve	21	12(5–41)	36	<0.001
Cyclins A and E				
Both +ve	12	44,5 (18–60)	42	
Either +ve	8	21(11–25)	14	<0.001
Both −ve	16	11 (5–41)	36	
Total	36			

For the endometrial cytology results, there is an inclination toward increased degree of severity with the increase in the time of exposure to TAM ($p = 0.09$) (Table 2). The Tamoxifen exposure duration for the endocervical cancer and atypia cases is 60 months.

A high significance was noticed between cyclins A and E positive samples and a longer Tamoxifen exposure duration/month (32 and 43 months respectively). The median Tamoxifen exposure duration for cases showing positivity for either A or E is 21 months, while for cases showing positivity for cyclins A and E, the median TAM exposure duration is 44.5 months. ($p < 0.001$) (Table 2).

According to Table 2 neither cyclin A nor E was detected before a median of 11 months.

Discussion

In 1989 Fornander provided early evidence that tamoxifen is a causal agent in the development of endometrial cancer, a 6.4-fold increase in the relative risk of developing endometrial cancer in a group of postmenopausal women with early breast cancer that was treated with adjuvant tamoxifen. The greatest risk occurred after five years in women who received 40 mg per day of tamoxifen [11]. Fisher in 1994 confirmed these findings when he reported the results of the National Surgical Adjuvant Breast and Bowel Project B-14 trial [12]. In 2009 Qureshi studied the incidence and type of endometrial abnormalities in long-term users of tamoxifen with breast cancer. [13].

Previously mentioned papers are in support to our present work, for endometrial cytology and this suggests an association between tamoxifen therapy and evidence of endometrial pathology of possible neoplastic potential.

Bland et al. studied 732 women with endometrial carcinoma, 59 patients (8%) had a previous diagnosis of breast cancer, of whom 29 (49%) had used tamoxifen. He also found that tamoxifen use for at least 60 months is associated with high

risk uterine histologic subtypes when compared to no tamoxifen use [14].

Further researches on a bigger sample of patients in Egypt are recommended to analyze the precise percentage of endometrial carcinoma in the general patient population versus those receiving TAM adjuvant therapy and the difference between their histo-pathological types.

Comparing the severity of endometrial changes in relation to tamoxifen exposure duration, we found an inclination toward increased degree of severity with the increase in TAM exposure duration. The endocervical cancer samples had the longest tamoxifen exposure duration (60 months) followed by the AGUS and active inflammation cases (18 and 17 months respectively) then, atrophy (15.5 months).

Previous studies showed that the risk of developing endometrial cancer is dose and time dependent [15]. Also, Bergman et al. explained that long term tamoxifen users had a worse prognosis of endometrial cancers, which seems to be due to less favorable histology and higher stage; this was confirmed by Prat, Jaime [16,17]. The same results were found by LE Donne where he found a significant correlation between the duration of TAM treatment and the severity of endometrial pathology. [18].

Our present study coincides and adds to previous existing data, suggesting a relationship between tamoxifen duration of use and development of endometrial carcinoma of more aggressive histologic types.

Progression through G_1-S transition and S phase of the cell cycle is mediated by cyclin-dependent kinase 2 (cdk2), which interacts with several cyclins. Two of these; cyclin E and cyclin A, are over expressed in many cancers. Expression and prognostic role of these cyclins in solid tumors is unknown [19].

In this study, we have analyzed the immune reactivity and prognostic relevance of the recently discovered cdk2-interacting cyclins A and E in the endometrium of estrogen receptor-positive (ER+) breast cancer patients receiving TAM adjuvant treatment.

Various methods are available for the measurement of proliferation rates in tumors, including mitotic counts, estimation of the fraction of cells in the S–phase of the cell cycle and immuno-histochemistry of proliferation-associated antigens. Proliferation rate can provide useful information on prognosis and aggressiveness of individual cancers and can be used to guide treatment in clinical practice.

Tsuda et al. studied the expression of cyclin D1, cyclin D2 and cyclin D3 in 10 normal endometrium, 18 endometrial hyperplasia and 35 endometrial carcinoma samples. There was no expression of the D1, D2 or D3 in both normal proliferative and hyper plastic samples while cancer cases had 28.6% D1 and 2.9% D2 nuclear localization and no expression (0%) of D3 [20]. Recent studies indicated that molecular markers are sensitive in the detection of the early changes in the carcinogenesis of the endometrium where there are many molecular abnormalities that are associated with the transformation of endometrium following tamoxifen administration [19]. Miranda et al. found that the use of TAM in postmenopausal women might be associated with an increased cellular proliferation in endometrial polyps without interfering angiogenesis or inactivation of tumor suppressor proteins.[21].

In the present study, a non-invasive method was employed to study the expression status of cyclin A and cyclin E that may influence the cell cycle mechanism and thus malignant potential. We studied the value of Cyclins A and E as biomarkers for determining the endometrial changes as a result of tamoxifen administration that may lead to malignancy.

Horrée et al. mentioned that cyclins A and E are expressed during the carcinogenesis of the endometrium and showed that expression of cyclin A and Ki67 gradually increased from normal through hyperplasia to carcinoma indicating their potential importance in both early and late carcinogenesis. Also he showed that cyclin E and P53 increased from hyperplasia to carcinoma underlining their role in late carcinogenesis [22].

Our data showed Immunoreactivity of the collected endometrial samples to both Cyclin-A (47.2%), and cyclin E (41.6%). Samples were immune reactive to A and/or E at different percentages depending on the cytological type of the endometrial change. The greatest percentage appeared in both endometrial carcinoma and atypia cases where they were 100% immunoreactive to A and E. Accordingly, cyclins A and E are expressed in precancerous and cancer cases which means that Cyclins A and E are expressed during the carcinogenesis of the endometrium as a result of tamoxifen administration.

A and E levels increase proportionate to duration of tamoxifen exposure. There was a highly significant correlation ($p < 0.001$) between cyclins A and E positivity and a longer Tamoxifen exposure duration. Neither cyclin A nor E was detected before a median time of 11 months.

We also found that tamoxifen exposure duration in cases showing positivity for both markers was significantly higher (44.5 months) than the duration in cases showing positivity for either cyclin A or E (21 month) .

Accordingly, the presence of both cyclins A and E together have a cumulative effect and emphasizes the possibility of malignant transformation. So follow up of cyclins positive cases at an earlier time is highly recommended, as the cyclin expression for these cases could be considered as a marker for the transformation.

It is very important to study when the cyclin over expression occurs within the cytological groups. For the AGUS group we found that the median time for cyclins A and E over expression was 20 and 35.5 months respectively. For the active inflammation it was 30 months for both A and E. For atrophy, it was 44 months for A and 43.5 for E.

These results might give us some information about the carcinogenesis process in the endometrium where AGUS type is formed after 18 months from tamoxifen administration; in some cases of the AGUS group, cyclin A is over expressed after 20 months while cyclin E is overexpressed after 35.5 months respectively. For the active inflammation type, it is formed after 17 months from tamoxifen administration, in some cases of this group cyclins A and E are over expressed after 30 months. The atrophy group is formed after 15.5 months from TAM administration, some cases of this group over express cyclins A and E after 44 and 43.5 months respectively. Finally malignant transformation occurs after 5 years.

So we can conclude that the cytological changes of the endometrium should be supported by over expression of cyclins A and E where their overexpression enforces the cells to go toward malignancy.

The routine use of endometrial biopsy of asymptomatic tamoxifen users failed to identify occult cancers [7].

Love et al. performed transvaginal ultrasounds on 357 asymptomatic patients with breast cancer who received tamoxifen. No significant lesions were detected in any of the women [23].

In this paper we studied the endometrial cytology using the Tao- brush non-invasive technique for discovering and diagnosing malignant and premalignant states of the endometrium according to the criteria described by DeMay [10].

Our results provide preliminary data to suggest that the immunocytology study of cyclins A and E may provide an initial surveillance tool in hormone receptor-positive breast cancer patients who were treated with tamoxifen based therapy. This method could provide increased sensitivity and specificity with reduced procedural risk and significant cost savings to the health care system.

Conclusion

Cyclins A and E expression is involved in the carcinogenesis of endometrium. The over expression of the cell cycle regulators (cyclins A and E) in the endometrial cells seems to appear after 11 months from the tamoxifen exposure. So follow up of the patients using these 2 markers is highly recommended starting from the 12th month. A special attention for cyclin positive cases is clearly needed, where the cyclin over expression in these cases may be an indicator for their transformation. For malignant transformation of the endometrium, the cytological changes should be supported by over expression of cyclins A and E where their overexpression enforces the cells to go toward malignancy.

Conflict of interest

No financial support or incentive has been provided for this article.

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